Transformation of *Clostridium Thermocellum* by Electroporation

Daniel G. Olson*,§ *and* Lee R. Lynd*,†,‡,§

Contents

Abstract

In this work, we provide detailed instructions for transformation of *Clostridium thermocellum* by electroporation. In addition, we describe two schemes for genetic modification: allelic replacement—where the gene of interest is replaced by an antibiotic marker and markerless gene deletion—where the gene of interest is removed and the selective markers are recycled. The

* Thayer School of Engineering at Dartmouth College, Hanover, New Hampshire, USA
† Mascoma Corporation, Lebanon, New Hampshire, USA
‡ Department of Biology, Dartmouth College, Hanover, New Hampshire, USA
§ BioEnergy Science Center, Oak Ridge, Tennessee, USA

markerless gene deletion technique can also be used for insertion of genes onto the *C. thermocellum* chromosome.

1. INTRODUCTION

Clostridium thermocellum has attracted attention due to its ability to rapidly solubilize cellulose. This ability appears to be due to a specialized multienzyme cellulase complex known as the cellulosome (Demain *et al.*, 2005). Since its first discovery (Lamed *et al.*, 1983), it has served as the model for complexed cellulase systems. Targeted gene deletion is a useful tool for understanding complex biological systems. Several improvements have been made to the *C. thermocellum* transformation protocol since it was first reported in our lab in 2004 (Olson, 2009; Tripathi *et al.*, 2010; Tyurin *et al.*, 2004). In addition, a variety of different schemes for gene disruption have been recently described (Argyros *et al.*, 2011; Olson *et al.*, 2010; Tripathi *et al.*, 2010). Here, we describe the transformation protocol currently in use in our lab and review the various engineering schemes that have been used for gene disruption.

Electrotransformation, the technique of introducing DNA into a cell by application of an electric field, is a technique that has existed for several decades. Most electrotransformation protocols require five steps: cell growth, harvest and washing, pulsing, recovery, and selection. Cells are typically harvested at some point in the growth curve between mid-log and early exponential phase. Then, cells are washed to remove the media, which typically contains charged ions that can interfere with electrical pulse application. Next, cells and DNA are mixed, and an electric field is applied. The electric field interacts with the cell membrane causing the temporary formation of holes (or pores) that allow the DNA to enter the cells. Pore size is affected by the amplitude and duration of the applied electrical field. Stronger field strength and longer duration leads to the formation of larger pores, which may allow DNA to enter the cell more readily; however, pores that are too large and may lead to cell death. There are several good references that describe the development and theory of the electrotransformation technique (Chang *et al.*, 1992; Dower et al., 1988).

2. MATERIALS

2.1. Media

A variant of DSM122 media referred to as CTFUD is used. The composition of this medium is described in Table 17.1. To prepare chemically defined medium (CTFUD-NY), yeast extract was replaced by a vitamin

Table 17.1 Recipe for CTFUD media

Reagent	FW (g/mol)	Amount (g/l)
Water (to 1L final volume)		
Sodium citrate tribasic dihydrate, $Na_3C_6H_5O_7 \cdot 2H_2O$	294.10	3.000
Ammonium sulfate, $(NH_4)_2SO_4$	132.14	1.300
Potassium phosphate monobasic, KH_2PO_4	136.09	1.500
Calcium chloride dihydrate, $CaCl_2 \cdot 2H_2O$	147.01	0.130
L–Cysteine–HCl	175.60	0.500
MOPS sodium salt[a]	231.20	11.560
Magnesium chloride hexahydrate, $MgCl_2 \cdot 6H_2O$	203.30	2.600
Ferrous sulfate heptahydrate, $FeSO_4 \cdot 7H_2O$	278.01	0.001
Cellobiose	342.30	5.000
Yeast extract		4.500
Resazurin 0.2% (w/v)		0.5 ml/l

[a] Adjust pH to 7.0 after addition of MOPS.

Table 17.2 Vitamin solution

25 ml $1000 \times$ vitamin solution recipe	
Component	Amount
Water (final volume) (ml)	25
Pyridoxamine HCl (mg)	50
Biotin (mg)	5
p-Aminobenzoic acid (PABA) (mg)	10
Vitamin B_{12} (mg)	5

solution (Table 17.2). The vitamin solution was originally developed by Johnson *et al.* (1981). For solid media, 0.8% (w/v) agar was added to the medium. Solid media can be stored at 55 °C after autoclaving to avoid solidification. Alternatively, it can be allowed to solidify at room temperature and remelted by microwaving.

Washing of cells during preparation for electrotransformation is performed with a wash buffer consisting of autoclaved reverse-osmosis-purified water with a resistivity of 18 MΩ cm. The purpose of autoclaving the wash buffer is to remove oxygen in addition to sterilization. Alternatively, an aqueous wash buffer containing 10% glycerol and 250 mM sucrose can be used.

2.2. Stock solutions of selective agents

1. *Thiamphenicol (Tm)*: 10 mg/ml stock solution in either dimethyl sulfoxide (DMSO) or a 50% ethanol:50% water mixture, used at a range of final concentrations from 5 to 48 μg/ml. *Note*: DMSO is used for experiments where added ethanol could interfere with results.
2. *Neomycin (Neo)*: 50 mg/ml stock solution in water, filter sterilized and used at 250 μg/ml final concentration.
3. *5-Fluoroorotic acid (5FOA)*: 100 mg/ml stock solution in DMSO (Zymo Research Inc., part number F9003), used at 500 μg/ml final concentration.
4. *5-Fluoro-2′-deoxyuradine (FUDR)* (Sigma, F0503): 10 mg/ml stock solution in water, filter sterilized and used at a final concentration of 10 μg/ml.
5. *8-Azahypoxanthine (8AZH)* (Acros Organics, 202590010): 50 mg/ml stock solution in 1 M NaOH or KOH, filter sterilized and used at a final concentration of 500 μg/ml.

2.3. Strains

C. thermocellum strain DSM1313 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) is used for routine transformations. Although we have previously reported transformation of *C. thermocellum* strain ATCC27405, we do not use it for routine transformation due to low and highly variable transformation efficiency.

2.4. Plasmids

Plasmid pMU102 (Fig. 17.1) (GenBank accession number JF423903) is derived from plasmid pNW33N (Olson, 2009), which is available from the Bacillus Genetic Stock Center (Cleveland, OH). It contains an origin of replication that functions in *C. thermocellum* and is the basis for all other replicating plasmids described here.

3. METHODS

3.1. Transformation protocol

1. Cell growth (anaerobic)
   a. Grow 50–500 ml of culture to an $OD_{600} = 0.6–1.0$. Stirring is optional, although not necessary.
   b. Place on ice for 20 min (optional).
   c. (Optional pause point)—at this point, cells can be divided into 50 ml plastic conical tubes and stored at −80 °C. To resume the protocol, thaw tubes at room temperature.

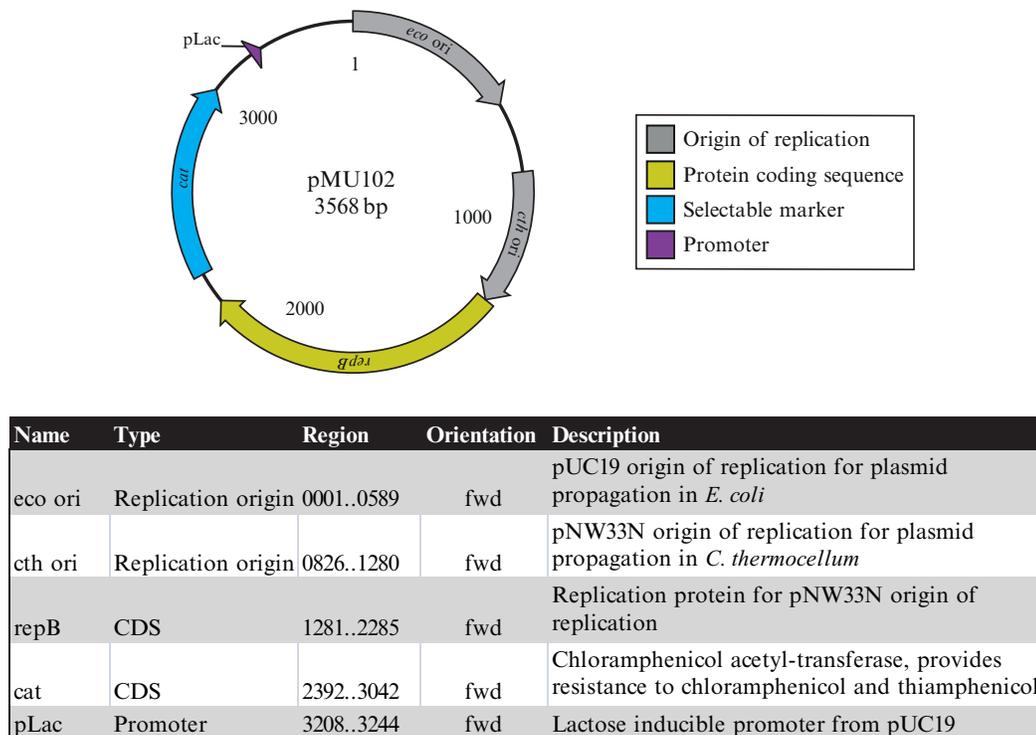| Name	Type	Region	Orientation	Description
eco ori	Replication origin	0001..0589	fwd	pUC19 origin of replication for plasmid propagation in *E. coli*
cth ori	Replication origin	0826..1280	fwd	pNW33N origin of replication for plasmid propagation in *C. thermocellum*
repB	CDS	1281..2285	fwd	Replication protein for pNW33N origin of replication
cat	CDS	2392..3042	fwd	Chloramphenicol acetyl-transferase, provides resistance to chloramphenicol and thiamphenicol
pLac	Promoter	3208..3244	fwd	Lactose inducible promoter from pUC19

Figure 17.1   Diagram of plasmid pMU102.

2. Cell harvesting and washing (aerobic). The goal of washing is to remove as much of the media as possible to ensure the resulting cell suspension has the required electrical properties (i.e., low conductivity). There is no need to resuspend the pellet during washes.
   a. Harvest cells by centrifugation (aerobic or anaerobic) at $6500 \times g$ for 12 min at 4 °C. *Note*: cell harvest and washing can also be performed at room temperature.
   b. Decant supernatant being careful not to disturb pellet. *Note*: we typically observe 5–15% of cells are lost during each decanting step.
   c. Refill centrifuge bottle with electroporation wash buffer without disturbing the pellet.
   d. Centrifuge at $6500 \times g$ for 12 min at 4 °C.
   e. Decant supernatant.
   f. Repeat wash (steps 2c–e) a second time.
3. Pulse application (anaerobic)
   a. Bring cells into an anaerobic chamber (we use Coy Laboratory Products Inc. vinyl anaerobic chambers).
   b. Resuspend cells with 100 μl anaerobic wash buffer by gentle pipetting.
   c. In a standard 1 mm electroporation cuvette, add 20 μl cell suspension and 1–4 μl DNA (100–1000 ng resuspended in water), mix by tapping the cuvette.

     d. Apply a square electrical pulse with an amplitude of 1500 V and duration of 1.5 ms (Bio-Rad Gene Pulser Xcell Microbial System part number 165-2662). *Note*: if arcing occurs, prepare a new cuvette and apply a pulse with amplitude decreased by 100 V. If arcing still occurs, repeat with yet another lower-amplitude pulse. Continue incrementally reducing amplitude until no arcing is detected.

4. Recovery (anaerobic)

     a. Resuspend pulsed cells in 3–5 ml media.

     b. Incubate at 51 °C for 6–18 h. Typically, we perform recovery in a stationary dry bath incubator. *Note*: an alternative recovery scheme is to plate cells directly onto selective media with no recovery period and incubate at 51 °C.

5. Select for transformants (anaerobic)

     a. Melt CTFUD agar (Table 17.1) and cool to ∼55 °C before addition of 6 μg/ml thiamphenicol. Mix 1 ml and 50 μl aliquots of recovered cells into 20 ml medium.

     b. Pour into Petri dish and allow to solidify at room temperature for 25–30 min.

     c. Incubate at 55 °C. *Note*: secondary containment is necessary to prevent plates from drying out (Remel/Mitsubishi 2.5L AnaeroPak Jar).

     d. Colonies should appear in 3–5 days.

3.2. Plasmid construction

Construction of plasmids is performed using standard cloning methods (Maniatis *et al.*, 1982) and yeast-mediated recombination (Shanks *et al.*, 2006, 2009). Selection in *Escherichia coli* is performed with 5 μg/ml chloramphenicol or 100 μg/ml carbenicillin, selection in yeast is performed with SD ura$^-$ medium according to the protocol described in Shanks *et al.* (2006).

3.3. Gene disruption by allelic replacement with a selective marker (Olson et al., 2010; Tripathi et al., 2010)

3.3.1. Plasmid design

Plasmids designed for gene disruption by allelic replacement have two regions that are homologous to regions flanking the gene target on the *C. thermocellum* chromosome. These homologous flanks should be 500–1000 bp in length. A positive-selectable marker is placed between the homologous flanks, and a negative-selectable marker is placed outside of the homologous flanks. In practice, we have used the *cat* marker driven by the *gapDH* promoter as the positive-selectable marker and *pyrF* driven by the *cbp* promoter as the negative-selectable marker. Use of this selection
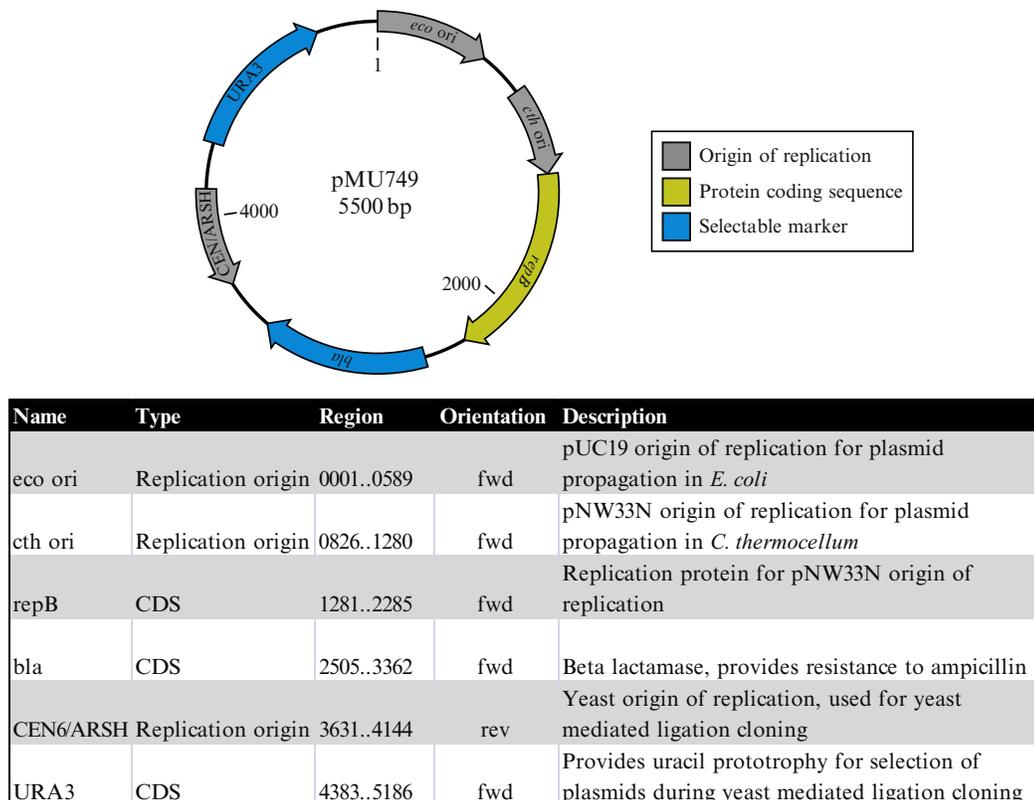
Name	Type	Region	Orientation	Description
eco ori	Replication origin	0001..0589	fwd	pUC19 origin of replication for plasmid propagation in *E. coli*
cth ori	Replication origin	0826..1280	fwd	pNW33N origin of replication for plasmid propagation in *C. thermocellum*
repB	CDS	1281..2285	fwd	Replication protein for pNW33N origin of replication
bla	CDS	2505..3362	fwd	Beta lactamase, provides resistance to ampicillin
CEN6/ARSH	Replication origin	3631..4144	rev	Yeast origin of replication, used for yeast mediated ligation cloning
URA3	CDS	4383..5186	fwd	Provides uracil prototrophy for selection of plasmids during yeast mediated ligation cloning

Figure 17.2   Diagram of plasmid pMU749.

requires a strain of *C. thermocellum* with an inactive *pyrF* gene. Plasmid pMU749 (GenBank accession number JN880474) is a backbone that, with the addition of an upstream (5′), a downstream (3′) flanking region, and the appropriate selectable markers, can be used for this gene disruption scheme (Fig. 17.2) (Olson *et al.*, 2010).

Cumulative marked genetic modifications via allelic replacement are limited by the number of positive–selectable markers available (currently three: *cat*, *neo*, and *pyrF*). Another limitation is that the insertion of a genetic marker onto the chromosome may affect expression of neighboring regions. For example, this scheme is unsuitable for deleting a single domain of a multidomain protein. The benefit of this scheme is that it requires fewer selection steps than markerless gene deletion.

3.3.2. Protocol for allelic replacement by Tm and 5FOA selection (Fig.17.3, allelic replacement, panels A–E)

1. Follow transformation protocol as described above.
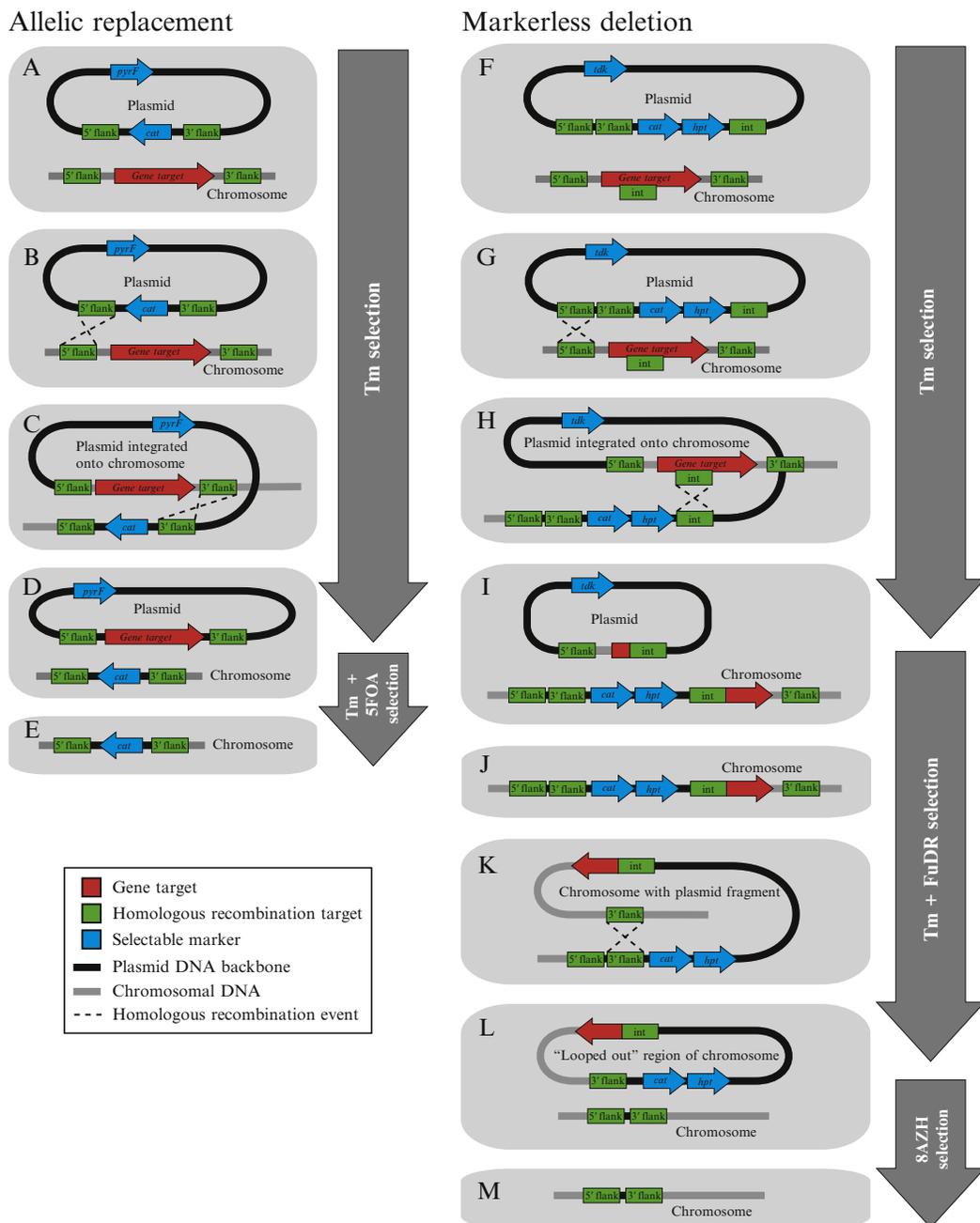2. Screen colonies by PCR to confirm presence of plasmid DNA.

Figure 17.3 Selection scheme diagram. The steps involved in gene disruption by allelic replacement or markerless gene deletion are described.

3. Inoculate confirmed plasmid–containing colony into liquid medium containing positive selection (i.e., Tm) (Fig. 17.3, panels B and C).
4. Plate four 100-fold serial dilutions of the culture on solid medium containing positive and negative selection such as Tm and 5FOA, respectively (Fig. 17.3, panels D and E).
5. Colonies should appear in 2–4 days.

6. Screen colonies by PCR to confirm the allelic replacement has been successful. *Note*: colony PCR is performed by resuspending 5 µl of a fresh colony in 100 µl water and using 0.02–2 µl of the resulting suspension as template for a PCR reaction. The PCR cycling protocol is modified to include a-10 min incubation at either 95 or 98 °C to lyse the cells.
7. Dilution plate to isolate individual colonies on media without selection. This final round of colony purification is recommended to ensure purity of colonies picked from within solid plates.

## 3.4. Markerless deletion using removable marker system (Argyros *et al.*, 2011)

3.4.1. Plasmid design

Plasmids designed for gene disruption by markerless deletion have two regions that are homologous to regions flanking the gene target on the *C. thermocellum* chromosome. These homologous flanks should be 500–1000 bp in length and are referred to as "5′ flank" and "3′ flank." Additionally, there is a third region with homology to the gene target. Typically, this region referred to as the "int region" is internal to the gene of interest and is also 500–1000 bp in length. *Note*: if the gene target is < 500 bp in length, the int region can overlap with the 5′ or 3′ flank. The 3′ flank is chosen from a region downstream of the gene target.

The 5′ and 3′ flanks are inserted on one side of the P*gapDH-cat-hpt* cassette, and the int region is inserted on the other side. The relative orientation of the 5′–3′ flanks and the int region affects the resulting intermediate chromosomal configuration. If the orientation of the flanks is the same as that shown in Fig. 17.3 (i.e., the order is 5′ flank, 3′ flank, cat, hpt, and int region), the intermediate chromosomal configuration will contain a version of the target gene missing the sequence *upstream* of the int region. The flanking regions can be placed in an alternate order (i.e., int region, cat, hpt, 5′ flank, and 3′ flank), and the intermediate chromosomal configuration will contain a version of the target gene missing the sequence *downstream* of the int region. In most cases, the difference between these two possibilities will be unimportant and in that case, we recommend placing the flanks in the order shown in Fig. 17.3. Note that orientations of homologous flanks must be in the same direction or the desired recombination events will not occur.

For this type of gene deletion strategy, we typically use a plasmid with a configuration similar to pAMG258 (Fig. 17.4) (GenBank accession number JN880475). Typically, the 5′ and 3′ flanking regions are inserted near the *Bam*HI site, and the int region is inserted near the *Asc*I site.

Although markerless gene deletion requires more rounds of selection than allelic replacement, it is currently considered the preferred method of chromosomal modification in *C. thermocellum*.
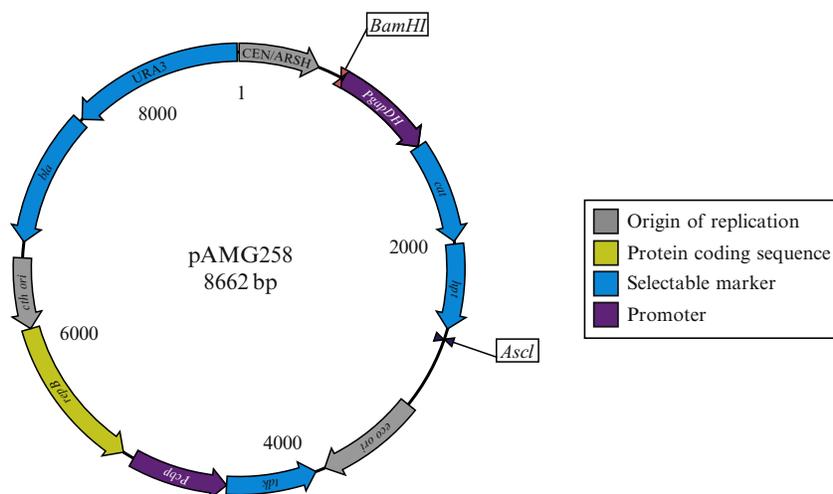
Name	Type	Region	Orientation	Description
CEN6/ARSH4	Replication origin	0001..0519	fwd	Yeast origin of replication, used for yeast mediated ligation cloning
PgapD	Promoter	0692..1339	fwd	Promoter from glyceraldehyde-3-phosphate dehydrogenase gene
cat	CDS	1340..1990	fwd	Chloramphenicol acetyl-transferase, provides resistance to chloramphenicol and thiamphenicol
hpt	CDS	2008..2553	fwd	Marker used for counterselection in *C. thermocellum* with 8AZH
eco ori	Replication origin	3099..3772	fwd	pUC19 origin of replication for plasmid propagation in *E. coli*
tdk	CDS	3833..4411	rev	Marker used for counterselection in *C. thermocellum* with FuDR
Pcbp	Promoter	4412..5032	rev	Promoter from cellobiose phosphorylase (cbp) gene of *C. thermocellum*
repB	CDS	5108..6112	rev	Replication protein for pNW33N origin of replication
cth ori	Replication origin	6113..6567	rev	pNW33N origin of replication for plasmid propagation in *C. thermocellum*
bla	CDS	6670..7530	rev	Beta lactamase, provides resistance to ampicillin
URA3	CDS	7548..8650	rev	Provides uracil prototrophy for selection of plasmids during yeast mediated ligation cloning

Figure 17.4 Diagram of plasmid pAMG258.

3.4.2. Protocol for markerless gene deletion by TM/FUDR/8AZH selection (Fig. 17.3, panels F–M)

1. Follow transformation protocol as described above (Fig. 17.3, panel F).
2. Screen colonies by PCR to confirm presence of plasmid DNA.
3. Inoculate confirmed plasmid-containing colony into liquid medium containing positive selection (i.e., Tm) (Fig. 17.3, panels G–I).
4. When the culture has grown up, plate four 100-fold serial dilutions on solid medium containing positive and first negative selection (Tm and FUDR, respectively) (Fig. 17.3, panel J).

5. Colonies should appear in 2–4 days.
6. Screen colonies by PCR to confirm first and second recombination events (i.e., Fig. 17.3, panels G and H).
7. Inoculate confirmed colony into liquid medium without selection.
8. When the culture has grown up, plate four 100-fold serial dilutions on solid medium containing the second negative selection (i.e., 8AZH) (Fig. 17.3, panels L and M).
9. Screen colonies by PCR to confirm the marker removal has been successful.
10. Dilution plate to isolate individual colonies on media without selection. This final round of colony purification is recommended to ensure purity of colonies picked from within solid plates.

3.5. Use of selective markers

1. *cat*—Preferred positive selection marker. Can be used with either CTFUD or CTFUD-NY media. Addition of Tm to the medium selects for the presence of a functional *cat* gene. Tm concentrations from 5 to 48 μg/ml are routinely used for selection, and increased concentrations have only a marginal effect on growth rate. See Olson thesis Figure 7 for details (Olson, 2011).
2. *neo*—Less-preferred compared with *cat*, since it inhibits growth at the concentrations required for selection. See Olson thesis Figure 14 for details (Olson, 2011). Can be used with either CTFUD or CTFUD-NY media. Addition of *neo* to the medium selects for presence of a functional *neo* gene.
3. *pyrF*—Can be used for both positive and negative selection in *ΔpyrF* backgrounds. Strains with nonfunctional *pyrF* gene need to be grown in media supplemented with at least 40 μg/ml uracil; however, excess uracil can inhibit growth (Tripathi *et al.*, 2010). This selection works best with actively growing cells in mid-log phase and with a final concentration of $<10^6$ cells/ml. *Positive selection*: Growth in CTFUD-NY media selects for presence of functional *pyrF* gene. *Negative selection*: Addition of 5FOA to CTFUD medium selects for the absence of a functional *pyrF* gene.
4. *tdk*—Preferred negative-selectable marker due to the ability to use it in the wild-type strain background. Either CTFUD or CTFUD-NY media can be used. Addition of FUDR to the medium selects for absence of a functional *tdk* marker. This selection works best with actively growing cells in mid-log phase and with a final concentration of $<10^6$ cells/ml.
5. *hpt*—Negative-selectable marker which requires a *Δhpt* background. Addition of 8AZH to CTFUD-NY medium selects for the absence of

the *hpt* marker. Since *C. thermocellum* contains a functional *hpt* gene (locus tag Cthe_2254 in the *C. thermocellum* strain ATCC 27405 genome), selection must be performed in a strain where this gene has been inactivated. This selection works best with actively growing cells in mid-log phase and with a final concentration of $< 10^6$ cells/ml.

4. Troubleshooting

4.1. Arcing during electric pulse application

1. Make sure DNA was eluted in water. Common DNA elution buffers such as EB and TE contain salts that can interfere with electroporation. We use the clean and concentrate kit for this purpose (Zymo Research Inc., part number D4003).
2. Reduce the quantity of DNA added.
3. Reduce pulse amplitude by 100 V and try pulsing a freshly prepared cuvette. If arcing is still observed, lower the amplitude and try again with a new cuvette. Repeat as necessary. Although transformation efficiency declines with decreasing pulse amplitude, transformation has been observed at amplitudes as low as 6 kV/cm (600 V pulse amplitude with a 1 mm cuvette).
4. Check resistivity of wash buffer, it should be 18 MΩ cm.
5. Make sure to remove as much media as possible during wash steps. Residual media may contain salts that can interfere with electroporation.

4.2. No colonies after transformation

1. Check to make sure recovery temperature is $< 51\,°C$, for details see Olson thesis Figure 3 (Olson, 2011).
2. Concentrate DNA. DNA should be at a concentration of > 200 ng/µl for best results.
3. If possible, use plasmid pMU102 as a positive control. We have occasionally observed sequence-dependent reductions in transformation efficiency. Plasmid pMU102 is known to transform *C. thermocellum* at high efficiency.

4.3. No colonies after selection with Tm and negative selection (5FOA, FUDR, or 8AZH)

1. Function of *cat* in single copy on the chromosome requires that it be driven by a strong promoter such as *gapDH* (Tripathi *et al.*, 2010). Check plasmid design and construction.

4.4. Colony PCR fails

1. If no PCR product was detected, try repeating the PCR with three 10-fold serial dilutions of the resuspended colony. Frequently PCR reactions fail as a result of *too much* cell material.
2. If the PCR generates a smear instead of a distinct band, try raising the annealing temperature. If you still get a smear, this may be the result of using an old culture. Try subculturing your cells and repeat the PCR with fresh culture.

4.5. Lawn of colonies after selection

1. All selections (except Tm) can be "overwhelmed" by plating concentrations of cells that are too high. Plate serial dilution to ensure a cell concentration to $< 10^6$ cells/ml is achieved.
2. CTFUD media occasionally develops a precipitate that can be mistaken for colony growth. If this is the case, an uninoculated plate can be prepared for comparison. To eliminate media precipitation, make sure components are added in the order listed. Also, when media is reheated, occasionally a precipitate forms that disappears upon cooling.
3. Confirm that genetic markers are still present in plasmid.

REFERENCES

Argyros, D. A., Tripathi, S. A., Barrett, T. F., Rogers, S. R., Feinberg, L. F., Olson, D. G., Foden, J. M., Miller, B. B., Lynd, L. R., Hogsett, D. A., and Caiazza, N. C. (2011). High ethanol titers from cellulose using metabolically engineered thermophilic, anaerobic microbes. *Appl. Environ. Microbiol.* **77,** 8288–8294.

Chang, D. C., Chassy, B. M., and Saunders, S. (1992). Guide to Electroporation and Electrofusion. Academic Press, San Diego, CA.

Demain, A. L., Newcomb, M., and Wu, J. H. D. (2005). Cellulase, clostridia, and ethanol. *Microbiol. Mol. Biol. Rev.* **69,** 124–154.

Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* **16,** 6127–6145.

Johnson, E. A., Madia, A., and Demain, A. L. (1981). Chemically defined minimal medium for growth of the anaerobic cellulolytic thermophile *Clostridium thermocellum. Appl. Environ. Microbiol.* **41,** 1060–1062.

Lamed, R., Setter, E., and Bayer, E. A. (1983). Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum. J. Bacteriol.* **156,** 828–836.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Olson, D. G. (2009). Electrotransformation of Gram-positive, Anaerobic, Thermophilic Bacteria. (W. I. P. Orginazation, ed.). Mascoma Corporation, Geneva, Switzerland.

Olson, D. G. (2011). Genetic investigations of the *Clostridium thermocellum* cellulosome. Thayer School of Engineering, Ph.D., Dartmouth College, Hanover, NH, 2011, p. 118.

Olson, D. G., Tripathi, S. A., Giannone, R. J., Lo, J., Caiazza, N. C., Hogsett, D. A., Hettich, R. L., Guss, A. M., Dubrovsky, G., and Lynd, L. R. (2010). Deletion of the Cel48S cellulase from *Clostridium thermocellum*. *Proc. Natl. Acad. Sci. USA* **107,** 17727–17732.

Shanks, R. M. Q., Caiazza, N. C., Hinsa, S. M., Toutain, C. M., and O'Toole, G. A. (2006). *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from gram-negative bacteria. *Appl. Environ. Microbiol.* **72,** 5027–5036.

Shanks, R. M. Q., Kadouri, D. E., MacEachran, D. P., and O'Toole, G. A. (2009). New yeast recombineering tools for bacteria. *Plasmid* **62,** 88–97.

Tripathi, S. A., Olson, D. G., Argyros, D. A., Miller, B. B., Barrett, T. F., Murphy, D. M., McCool, J. D., Warner, A. K., Rajgarhia, V. B., Lynd, L. R., Hogsett, D. A., and Caiazza, N. C. (2010). Development of pyrF-based genetic system for targeted gene deletion in *Clostridium thermocellum* and creation of a pta mutant. *Appl. Environ. Microbiol.* **76,** 6591–6599.

Tyurin, M. V., Desai, S. G., and Lynd, L. R. (2004). Electrotransformation of *Clostridium thermocellum*. *Appl. Environ. Microbiol.* **65,** 883–890.